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recA-Based PCR Assay for Accurate Differentiation of *Streptococcus pneumoniae* from Other Viridans Streptococci[▽]

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Proper identification of *Streptococcus pneumoniae* by conventional methods remains problematic. The discriminatory power of the 16S rRNA gene, which can be considered the "gold standard" for molecular identification, is too low to differentiate *S. pneumoniae* from closely related species such as *Streptococcus pseudopneumoniae*, *Streptococcus mitis*, and *Streptococcus oralis* in the routine clinical laboratory. A 313-bp part of *recA* was selected on the basis of variability within the *S. mitis* group, showing <95.8% interspecies homology. In addition, 6 signature nucleotides specific for *S. pneumoniae* were identified within the 313-bp *recA* fragment. We show that *recA* analysis is a useful tool for proper identification to species level within the *S. mitis* group, in particular, for pneumococci.

Streptococcus pneumoniae is the most common cause of community-acquired pneumonia and is also associated with bacteremia, meningitis, otitis media, and sinusitis (34). *S. pneumoniae* is a member of the *Streptococcus mitis* group, which currently includes *Streptococcus mitis*, *Streptococcus pseudopneumoniae*, *Streptococcus oralis*, *Streptococcus infantis*, *Streptococcus sanguinis*, *Streptococcus parasanguinis*, *Streptococcus cristatus*, *Streptococcus gordonii*, *Streptococcus peroris*, *Streptococcus australis*, *Streptococcus oligofermentans*, and *Streptococcus sinensis* (21, 44).

Clinical laboratories must be able to accurately differentiate *S. pneumoniae* from other viridans streptococci commonly found in clinical samples to facilitate appropriate antimicrobial therapy. Discrimination of *S. pneumoniae* from closely related species such as *S. pseudopneumoniae*, *S. oralis*, and *S. mitis* remains problematic since conventional phenotypic methods like colony morphology, bile solubility, and optochin susceptibility testing, as well as commercial systems (API 20 Strep and Vitek 2; bioMérieux, Marcy l'Etoile, France), do not always provide accurate identification (3, 5, 15, 18, 34) and often lead to misidentification. Moreover, sequence analysis of the 16S rRNA gene, a method widely used for bacterial identification to species level (4–8, 37), is not sufficiently discriminative (23). Several studies proposed the analysis of additional, more discriminative target genes like *sodA* (3, 24, 36), *rpoB* (12, 21), *gdh* (21, 35), and *groEl* (16) to differentiate species within the *S. mitis* group. However, an accurate differentiation from the more recently described *S. pseudopneumoniae*, which is closely related to *S. pneumoniae*, was either not demonstrated (3) or not investigated in detail (16, 35).

Other PCR-based approaches for accurate identification of *S. pneumoniae* rely on the detection of pneumococcal toxins or virulence factors, such as the pneumolysin (*ply* gene) and autolysin (*lytA* gene) (28, 33, 40), which are usually not present in other alpha-hemolytic streptococci. The usefulness of such assays is questionable, as false-positive results due to cross-reactivity among *S. mitis*, *S. oralis*, or *S. pseudopneumoniae* strains were generated (1, 3, 14, 17, 45).

Phylogenetic analysis of *recA*, encoding the highly conserved subunit of the bacterial recombinase, proved to be a valuable tool for bacterial species assignment (13, 30, 31, 42, 46) but has not been investigated in-depth for species differentiation of the *S. mitis* group.

The aim of this study was to assess *recA* as a gene target for proper identification of streptococci, particularly *S. pneumoniae*. We identified a 313-bp *recA* fragment that differentiates members of the *S. mitis* group and enables accurate assignment to species level.

MATERIALS AND METHODS

Bacterial strains. Type strains of *S. pneumoniae* (NCTC 7465), *S. mitis* (NCTC 12261), and *S. oralis* (NCTC 11427) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). Type strains of *S. pseudopneumoniae* (ATCC BAA-960), *S. infantis* (ATCC 700779), and *S. oligofermentans* (LMG 21535) were obtained from the Institut Pasteur (Paris, France). Other streptococcal strains used in this study were isolated from clinical samples (blood cultures or other normally sterile body sites) in our laboratory: (i) 11 isolates collected from January to April 2009 and (ii) 20 isolates previously analyzed (5). Streptococcal strains were routinely cultured on sheep blood agar. Phenotypic characterization included colony morphology, susceptibility to optochin, bile solubility, and capsular serotyping (National Centre for Invasive Pneumococci, Institute for Infectious Diseases, University Bern, Bern, Switzerland).

***recA* sequence analysis.** DNA was extracted from the cultures as follows. A loopful of bacteria was suspended in 500 μl 0.9% NaCl and incubated by shaking at 80°C for 10 min. After centrifugation, the pellet was resuspended in 200 μl of InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and incubated at 56°C for 2 h and subsequently at 95°C for 10 min. The mixture was centrifuged and the supernatant was used as the template for PCR.

For amplification, primers *recA* 2F [5′-GCCTT(T/C)ATCGATGC(C/T/G)GA(G/A)CA-3′] and *recA* 5R [5′-GTTTCCGG(G/A)TT(A/T/G)CC(G/A)AACAT-3′] were used. PCR cycling parameters included an initial denaturation for 5 min at 95°C; 40 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; and a final extension for 10 min at 72°C. Five microliters of the DNA extract was used for amplification in a total volume of 50 μl containing 1.25 U of FastStart *Taq* DNA polymerase (Roche Diagnostics, Rotkreuz, Switzerland) and the appropriate buffer. Amplicons were purified with a QIAquick PCR purification kit

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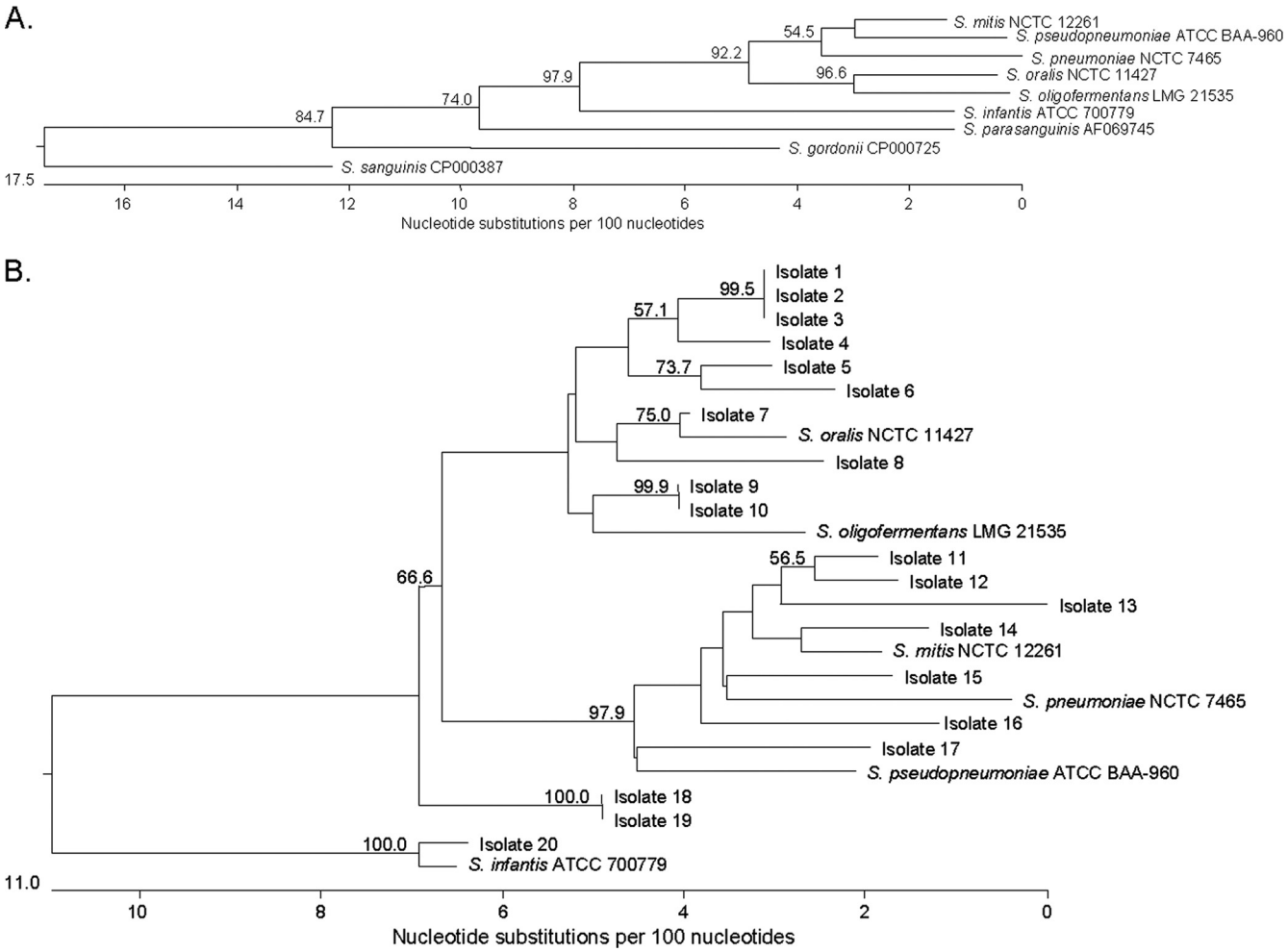

FIG. 1. Homology analysis of *recA* (313-bp fragment) within the *Streptococcus mitis* group. (A) Phylogenetic tree of 313-bp *recA* sequences of *Streptococcus mitis* group strains. Validated *recA* sequences of *S. peroris*, *S. australis*, *S. cristatus*, and *S. sinensis* were not represented in GenBank. Bootstrap values were calculated from 1,000 trees; only values exceeding 50% are shown. (B) Phylogenetic tree of 313-bp *recA* sequences of *Streptococcus* type strains and *Streptococcus* clinical isolates ($n = 20$). Bootstrap values were calculated from 1,000 trees; only values exceeding 50% are shown.

(Qiagen AG, Hombrechtikon, Switzerland) and were sequenced with forward primer *recA* 2F and reverse primer *recA* 5R by use of a BigDye kit and an automatic DNA sequencer (ABI Prism 3100 genetic analyzer; Applied Biosystems, Zug, Switzerland). The sequences were edited using the software program Megalign Lasergene (version 7; DNAStar Inc., Madison, WI). Distances of the *recA* sequences were calculated by using Smartgene software (Zug, Switzerland). Multiple alignment of the sequences was performed with the Clustal V program (20) (Megalign Lasergene, version 7), and construction of a phylogenetic tree was performed with the neighbor-joining method (38).

Nucleotide sequence accession numbers. The *recA* sequences of strains *S. pneumoniae* NCTC 7465^T, *S. mitis* NCTC 12261^T, *S. oralis* NCTC 11427^T, *S. pseudopneumoniae* ATCC BAA-960^T, *S. infantis* ATCC 700779^T, *S. oligofermentans* LMG 21535^T, and clinical isolates of *S. pneumoniae* have been group deposited in GenBank under BankIt 1363616 (with individual numbers HM572273, HM572274, HM572275, HM572276, HM572277, HM572278, HM572279, HM572280, HM572281, HM572282, HM572283, HM572284, HM572285, HM572286, HM572287, HM572288, and HM572289).

RESULTS

Selection of a hypervariable *recA* fragment for differentiation of *Streptococcus mitis* group members. To identify a small hypervariable region suitable for differentiation and efficient PCR amplification, we aligned complete *Streptococcus recA* sequences available in GenBank (accession numbers are given in parentheses), e.g., *Streptococcus pyogenes* (NC_009332), *S. pneumoniae* (NC_008533), *S. gordonii* (CP000725), *S. parasanguinis* (AF069745), *S. sanguinis* (CP000387), *Streptococcus mutans* (NC_004350), and *Streptococcus agalactiae* (NC_004116). *recA* genes of *Pseudomonas aeruginosa* (NC_002516) and *Escherichia coli* (NC_002695) were used as outlier sequences. An internal 313-bp *recA* fragment including hypervariable regions was selected for amplification with consensus *recA* PCR primers 2F and 5R (see Materials and Methods). This region corresponds to *Escherichia coli recA* positions 294 to 606 (NC_002695 [19]).

Homology analysis of *recA* within *Streptococcus mitis* group. Phylogenetic analysis of the 313-bp *recA* fragment of the *S. mitis* group strains was made to determine its differentiating ability (Fig. 1A). *recA* sequences were generated from *S. mitis* group type strains (i.e., *S. pneumoniae* NCTC 7465^T, *S. mitis* NCTC 12261^T, *S. oralis* NCTC 11427^T, *S. pseudopneumoniae* ATCC BAA-960^T, *S. infantis* ATCC 700779^T, and *S. oligofermentans* LMG 21535^T) or were obtained from published se-

TABLE 1. Homology analysis of partial *recA* of *Streptococcus* sp. type strains

Species	Homology of the 313-bp *recA* fragment (%)			
	S. pneumoniae	*S. mitis*	*S. oralis*	*S. pseudopneumoniae*
S. pneumoniae	100	95.2	91.7	93.3
S. mitis		100	91.7	95.8
S. oralis			100	92.0
S. pseudopneumoniae				100

quences from GenBank (*S. parasanguinis*, accession number AF069745; *S. gordonii*, accession number CP000725, *S. sanguinis*, accession number CP000387). Partial *recA* sequence analysis revealed homologies of <95.8% between species (Table 1).

Intraspecies variability of *Streptococcus pneumoniae recA*. A set of 11 published *recA* sequences of *S. pneumoniae* strains available from GenBank (CP000410 [29], NC_008533 [29], AE005672 [41], CP001015 [11], NC_011072 [11], AE007317 [22], NC_003098 [22], CP001033 [10], NC_010582 [10], NC_003028 [41], FM211187 [9]) and 11 *recA* sequences obtained from accurately assigned *S. pneumoniae* clinical isolates from our laboratory were analyzed for homology to determine the intraspecies variability in the *recA* fragment sequence. The 22 sequences showed >99.7% identity.

Proof of principle: identification of viridans streptococci on the basis of sequence analysis of partial *recA* fragment. From 20 clinical isolates of viridans streptococci, previously unidentifiable to species level by 16S rRNA gene sequencing and with the API 20 Strep system (bioMérieux) (5), the 313-bp *recA* fragment was amplified and analyzed for sequence homology. For each isolate, a sequence homology of ≤95.5% to the *recA* sequence of the *S. pneumoniae* type strain was observed (Table 2). For 12 isolates (numbers 5, 6, 7, 8, 12, 13, 14, 15, 16, 18, 19, and 20), *recA* analysis yielded an assignment to species level (Table 2). *recA* sequence similarities to the best taxon ranged from 95.5% to 99.0%, with a difference of ≥1.0% to the next best taxon. For the other eight isolates (numbers 1, 2, 3, 4, 9, 10, 11, and 17), best matches were observed with *S. oralis*/*S. oligofermentans* and *S. mitis*/*S. pseudopneumoniae*, respectively.

A combined phylogenetic analysis of the *recA* sequences of the clinical isolates and type strains showed two major clusters: one containing the type strains of *S. mitis*, *S. pneumoniae*, and *S. pseudopneumoniae* and the other containing the type strains of *S. oralis* and *S. oligofermentans* (Fig. 1B). Within the *S. mitis*/*S. pneumoniae*/*S. pseudopneumoniae* cluster, the lineage containing the *S. pneumoniae* type strain branched off into a tight subcluster.

Signature nucleotides in *Streptococcus pneumoniae recA*. The *recA* sequences of type strains *S. pneumoniae* NCTC 7465, *S. pseudopneumoniae* ATCC BAA-960, *S. mitis* NCTC 12261, *S. oralis* NCTC 11427, and all clinical isolates used in this study ($n = 31$) and published *recA* sequences of *S. pneumoniae* from GenBank ($n = 11$) were aligned to detect specific positions within the 313-bp *recA* fragment that distinguished *S. pneumoniae* (molecular signatures). The alignment showed 6 bp specific for *S. pneumoniae* at positions 97, 160, 199, 247, 250, and 280 (Table 3).

DISCUSSION

Accurate identification to species level of *S. mitis* group members is of clinical importance, since this group contains

TABLE 2. *recA* sequence-based identification of viridans streptococcal clinical isolates[a]

Clinical isolate	No. of mismatches/total no. of nucleotides (% sequence similarity)				Assignment to species level
	S. mitis	*S. oralis*	*S. pneumoniae*	*S. pseudopneumoniae*	
1	25/313 (92.0)	15/313 (95.2)	28/313 (91.1)	24/313 (92.3)	*S. oralis*[b]
2	25/313 (92.0)	15/313 (95.2)	28/313 (91.1)	24/313 (92.3)	*S. oralis*[b]
3	25/313 (92.0)	15/313 (95.2)	28/313 (91.1)	24/313 (92.3)	*S. oralis*[b]
4	25/313 (92.0)	15/313 (95.2)	28/313 (91.1)	22/313 (93.0)	*S. oralis*[b]
5	26/313 (91.7)	12/313 (96.2)	32/313 (89.8)	25/313 (92.0)	*S. oralis*
6	25/313 (92.0)	13/313 (95.8)	31/313 (90.1)	24/313 (92.3)	*S. oralis*
7	22/313 (93.0)	4/313 (98.7)	23/313 (91.7)	21/313 (93.3)	*S. oralis*
8	29/313 (90.7)	11/313 (96.5)	31/313 (90.1)	28/313 (91.1)	*S. oralis*
9	22/313 (93.0)	10/313 (96.8)	24/313 (92.3)	21/313 (93.3)	*S. oralis*[c]
10	22/313 (93.0)	10/313 (96.8)	24/313 (92.3)	21/313 (93.3)	*S. oralis*[c]
11	10/313 (96.8)	25/313 (92.0)	14/313 (95.5)	11/313 (96.5)	*S. mitis*/*S. pseudopneumoniae*
12	9/313 (97.1)	27/313 (91.4)	16/313 (94.9)	13/313 (95.8)	*S. mitis*
13	12/313 (96.2)	27/313 (91.4)	21/313 (93.3)	21/313 (93.3)	*S. mitis*
14	7/313 (97.8)	27/313 (91.4)	14/313 (95.5)	18/313 (94.2)	*S. mitis*
15	10/313 (96.8)	26/313 (91.7)	15/313 (95.2)	13/313 (95.8)	*S. mitis*
16	13/313 (95.8)	29/313 (90.7)	21/313 (93.3)	18/313 (94.2)	*S. mitis*
17	16/313 (94.9)	24/313 (92.3)	17/313 (94.6)	15/313 (95.2)	*S. mitis*/*S. pseudopneumoniae*
18	21/313 (93.3)	18/313 (94.2)	25/313 (92.0)	20/313 (93.6)	*S. oligofermentans*[d]
19	21/313 (93.3)	18/313 (94.2)	25/313 (92.0)	20/313 (93.6)	*S. oligofermentans*[d]
20	41/313 (86.9)	37/313 (88.2)	47/313 (85.0)	37/313 (88.2)	*S. infantis*[e]

[a] Homology analysis between the partial *recA* sequences obtained from viridans streptococcal clinical isolates ($n = 20$) and type strains of *S. mitis*, *S. oralis*, *S. pneumoniae*, and *S. pseudopneumoniae* was performed.
[b] 95.2% sequence homology to *S. oligofermentans* was found.
[c] 96.8% sequence homology to *S. oligofermentans* was found.
[d] Fourteen mismatches/313 nucleotides (95.5%).
[e] Three mismatches/313 nucleotides (99.0%).

TABLE 3. Signature nucleotides specific for *Streptococcus pneumoniae* observed in 313-bp *recA* fragment[a]

Species	Nucleotide at the following position in 313-bp *recA* fragment:					
	97	160	199	247	250	280
S. pneumoniae	G	T	T	C	C	T
S. mitis	A	C	C	T	T	C
S. oralis	T	A	C	A	T	C
S. pseudopneumoniae	A	A	C	T	T	C

[a] Signature nucleotides are based on homology analyses of *recA* sequences from (i) type strains of *S. pneumoniae*, *S. pseudopneumoniae*, *S. mitis*, and *S. oralis*, (ii) 11 clinical *S. pneumoniae* isolates characterized in depth, (iii) 11 *S. pneumoniae* GenBank entries (all published), and (iv) 20 clinical isolates of viridans streptococci previously unidentifiable to the species level by 16S rRNA gene sequencing and with the API 20 system (Table 2).

pathogens, e.g., *S. pneumoniae*, and commensals of the human oral cavity, such as *S. mitis* and *S. oralis* (34). In view of increasing resistance to penicillin and macrolide antibiotics (2, 34), proper identification of viridans streptococci is important for antimicrobial therapy. Differentiation between *S. pneumoniae* and *S. pseudopneumoniae* is of relevance, as those isolates are assumed to be involved in the exacerbation of chronic obstructive pulmonary disease (25).

We have selected a 313-bp *recA* fragment that shows a significant variability among the *S. mitis* group members (Fig. 1; Table 1) and identified molecular signatures confirming accurate identification of pneumococci (Table 3). Implementation of the presented *recA* PCR assay in routine laboratory diagnostics is facilitated by the fact that a small part of a single gene is sufficient for accurate assignment of *S. pneumoniae.*

For assignment to species level using analysis of housekeeping genes, e.g., *recA*, approved criteria such as those for the 16S rRNA gene (5) are not available. Sequence homologies of more than 94% to 95% with a reference sequence was proposed to be appropriate for identification to species level for housekeeping genes such as *rpoB* (26, 32) or *recA* (43). This is in agreement with our data: most species showed sequence identity of more than 95.5% to the best taxon match, with a demarcation of ≥1.0% to the next homologous taxon (Table 2).

As proof of principle, we retrospectively analyzed a number of clinical isolates ($n = 20$) which remained unidentified to species level by phenotypic methods and 16S rRNA gene sequencing in routine diagnostics (5). For all isolates investigated, differentiation from *S. pneumoniae* was achieved by partial *recA* sequence analysis (≤95.5% sequence similarity) and from nucleotide signatures (Table 3). Twelve strains were identified to species level and eight strains were assigned to *S. oralis*/*S. oligofermentans* ($n = 6$) and *S. mitis*/*S. pseudopneumoniae* ($n = 2$), respectively. A close relation of *S. oligofermentans* to *S. oralis* was previously observed by analysis of the *groEL* gene (16). However, 16S rRNA gene analysis can accurately differentiate *S. oligofermentans* and *S. oralis* (data not shown). Despite a *recA* sequence similarity of 95.8% between the type strains of *S. mitis* and *S. pseudopneumoniae*, accurate differentiation between these species was not shown for two clinical isolates. Thus, in routine diagnostics, *recA* analysis is a valuable tool for identification of pneumococci, but limitations on discrimination of other members of the *S. mitis* group were observed.

As described earlier by analysis of other targets (24, 27, 35, 36), we observed heterogeneity within the *S. mitis* and *S. oralis* cluster (Fig. 1B) but a tight homogeneity of the *S. pneumoniae* strains. The hypothesis of a common ancestor of *S. mitis*, *S. pneumoniae*, and *S. pseudopneumoniae* proposed previously (27) is supported by our data. Our results show that the heterogeneity of *S. oralis* and *S. mitis* strains (Fig. 1B; Table 2) as well as the homogeneity of *S. pneumoniae* strains occurs not only in reference strains (35) but also in clinical isolates.

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